REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED FINAL 01 Feb 91 TO 31 Jan 93

4. TITLE AND SUBTITLE ORGANIZATION OF THE HUMAN CIRCADIAN SYSTEM	5. FUNDING NUMBERS AFOSR-91-0175 61102F 2312 CS
6. AUTHOR(S) Dr Robert Y. Moore	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dept of Psychiatry Univ of Pittsburgh W1656 Biomedical Science Tower	8. PERFORMING ORGANIZATION REPORT NUMBER AFOSR-TR- 93 0336
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) AFOSR/NL 110 Duncan Avenue, Suite B115 Bolling AFB DC 20332-0001	10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release;
distribution unlimited

13. ABSTRACT (Maximum 200 words)

In brains obtained from late gestation fetuses (33-36 weeks), newborns and young individuals to approximately age 50, the SCN is virtually always identifiable as a discrete nucleus with clear boundaries. From age 50-90, it is sometimes evident and and sometimes not evident in the material. We have completed analysis of 22 hypothalami prepared for immunocytochemistry, including quantitative analysis. Sections are routinely stained for VIP, VP, NPY and NT. This analysis has revealed several interesting aspects of the human SCN. First, in contrast to what is found in Nissl material, the SCN is always evident as a distinct nucleus in immunocytochemical material. Second, it appears as the first component of the hypothalamus to be found in a rostrocaudal set of coronal sections. Third, the human SCN is characterized by four separate populations of neurons that have different peptide content. These neuronal populations have a different distribution in the nucleus. In contrast to all other mammals, the human SCN contains a population of NPY + neurons that overlaps the VIP + group but extends dorsally beyond it in the center on the SCN. Among the NPY + neurons are scattered coarse fibers and varicosities and a fairly dense plexus of very fine fibers and samll varicosities. These are very similar in morphology to

14. SUBJECT TERMS	15. NUMBER OF PAGES
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
(U)	(U)	(U)	(UL)

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

FINAL TECHNICAL REPORT

"ORGANIZATION OF THE HUMAN CIRCADIAN SYSTEM"

Principal Investigator:
Robert Y. Moore, M.D., Ph.D.
Department of Psychiatry
University of Pittsburgh
Pittsburgh, PA 15261

Project Period: 10/1/89-1/31/93

Air Force Office of Scientific Research
Bolling Air Force Base, D.C. 20332-6448
Attention: Dr. Genevieve Haddad

TECHNICAL REPORT

Objectives: The overall objective of this project is to provide a detailed description of the organization of the primate, particularly human, circadian timing system. The specific objectives were as follows: 1) Analyze retinal projections to the anterior hypothalamus, including the suprachiasmatic nucleus (SCN), in the macaque monkey; 2) Provide an immunocytochemical analysis of the monkey lateral geniculate complex; 3) Provide an immunocytochemical analysis of the monkey SCN; 4) Provide an immunocytochemical analysis of the human lateral geniculate; 5) Provide an analysis of human retinohypothalamic projections using DiI; 6) Provide an analysis of the human SCN using cell stains and immunocytochemistry. 7) Analyze SCN efferents in the monkey and human using vasoactive intestinal polypeptide immunocytochemistry.

Research Accomplishments: These will be described for each of the objectives above.

1. Introduction - General Considerations. This program was funded in November, 1989. In November, 1990, I moved to the University of Pittsburgh and the research was interrupted for approximately 4 months. The move to Pittsburgh had several advantages: 1) the laboratory facilities are much superior to those in Stony Brook; 2) I am free of clinical responsibilities and have much reduced administrative duties making more time available for research; 3) there is a greater availability of human material in Pittsburgh. The time in Pittsburgh has been quite productive and this will be detailed below.

There are some comments about the availability of tissue that should be made here. We have access to as many as 4 brains a week from the routine autopsy service. This has the advantage, since we cannot process more than one brain a week, of allowing selection of the best fixed normal material. I should emphasize, however, that this is material obtained with postmortem intervals between death and immersion fixation of the brain of 4-48 hours. The brains are fixed in 10% buffered formaldehyde for two weeks and we then obtain them at routine brain cutting done by the Division of Neuropathology. In the last year we have begun obtaining brains from a separate source, the Coroner of Allegheny County through the Suicide Center directed by Dr. John Mann, Department of Psychiatry. These brains, often from relatively young individuals, are obtained fresh with short (2-4 hour) postmortem intervals and the hypothalamus is delivered directly to us for fixation and analysis. It has become clear that this material is more useful than that obtained from the autopsy service in three ways. First, it has much better VIP immunoreactivity and, hence, will permit a more detailed analysis of SCN efferents. Second, the material generally has better axonal morphology, particularly fine axons, than the material from the autopsy service. Third, Dr. Mann's group performs "psychological autopsies" in which detailed historical information is obtained from the families of suicide victims and controls. This provides the potential for obtaining information about circadian function for individuals for whom we have detailed morphological information. This is not to imply that the autopsy service material is not useful and informative. We obtain very few hypothalami, and no lateral geniculates, from the Suicide Center. The autopsy service is and will remain, our major source of material.

In addition to the human material, we have obtained macaque monkey material from Dr. Anita Hendrickson, Washington Regional Primate Center, University of Washington, Seattle, and from Dr. David Lewis, Department of Psychiatry and Pittsburgh Primate Research Center. This has consisted of approximately 8 well-fixed hypothalami from adult monkeys that have been sacrificed for other projects carried on by the investigators note above. This material has been used for immunohytochemical analysis of the SCN. In addition, we have purchased two adult monkeys for investigation of retinal afferents to the hypothalamus and thalamus. The cost of a monkey now is about $2000, greatly limiting our capacity in this area.

2. Monkey Retinal Projections. In two adult macaque monkeys, we have injected cholera toxin conjugated to HRP into one eye and sacrificed the animals after a one week survival, and processed sections through the hypothalamus, thalamus and upper midbrain using the HRP method. Projections to the SCN are very similar in distribution to those in the rat with one major exception. In the monkey, the retinohypothalamic projection is approximately twice as great to the ipsilateral SCN as to the contralateral nucleus. This is predominantly to the ventral SCN where it forms a dense plexus of terminals largely overlapping the distribution of VIP+ neurons. There are scattered RHT axons to the medial preoptic area, the dorsal SCN, the anterior hypothalamic area adjacent to the SCN with a group of axons extending rostrally and laterally along the ventral surface of the brain to enter the anterior amygdaloid area. There is a significant projection to the retrochiasmatic area that extends caudal to the SCN. There is also a significant projection to the lateral hypothalamic area which is greater on the contralateral than the ipsilateral side.

In the lateral geniculate complex, there is a very dense projection to the DLG which has the typical pattern of laminar organization (to layers 1,4 and 6 contralateral, and 2, 3 and 5 ipsilateral to the injected eye). On cytoarchitectonic analysis, the pregeniculate nucleus (cf Jones, 1985, for review), has two evident subdivisions. The largest of these, located medial and dorsal to the DLG, consists of medium-sized neurons in a relatively homogeneous population that has two apparent subdivisions, a small one dorsal and lateral to the DLG and a larger one predominantly dorsomedial to the DLG and extending over the top of the cerebral peduncle to be continuous with the zona incerta. We will refer to these as the dorsolateral and dorsomedial subdivisions, respecting. The other major subdivision of the pregeniculate nucleus is a group of cells that lies dorsal and lateral to the DLG. This is comprised of larger, more darkly stained neurons than those in the region noted above. These occur in small groups along the lateral surface of the DLG and a larger group dorsally that is continuous with the thalamic reticular nucleus. This nucleus appears to be the primate homologue of the perigeniculate nucleus of the cat (cf Jones, 1985). Retinal projections to the pregeniculate nucleus have the following pattern. Projections to the dorsomedial subdivision are bilateral with those to the contralateral side approximately twice as dense as those to the ipsilateral side. In the dorsolateral subdivision, however, the projections are almost exclusively to the contralateral side. On this basis, we conclude that this division is the primate homologue of the VLG and that the larger, medial subdivision is the homologue of the IGL. This suggests that the IGL remains an important component of the geniculate complex in primates whereas the VLG appears much reduced in relative size.

3. Monkey Lateral Geniculate - Immunocytochemistry. The pregeniculate extends from the rostral pole of the geniculate complex to nearly the caudal pole. We have examined the nucleus with antisera to NPY, ENK and SP. NPY+ neurons are present throughout the medial subdivision, both in the putative VLG and IGL homologue. These are relatively evenly distributed and associated with a dense axonal plexus that extends medially to become continuous with a similar plexus in the zona incerta. There is a nearly identical ENK+ plexus and occasional ENK+ neurons are evident. These are substantially fewer than the NPY+ neurons but this is not colchicine-treated material and the differences may reflect differences in perikaryal peptide content, differences in relative sensitivity of the antisera, or both, rather than a true difference in neuron number. We do not find SP+ neurons in the pregeniculate nucleus but we do note a dense plexus in the dorsomedial subdivision with a less dense plexus in the dorsolateral subdivision, consistent with the view that these are IGL and VLG homolgues, respectively. We do not observe immunoreactive elements in the perigeniculate homologue with any of these antisera.

4. <u>Macaque Monkey SCN.</u> The SCN in the monkey is very similar in appearance in Nissl stains to that in the rat. It is a compact nucleus containing small neurons and lies dorsal to the optic chiasm and lateral to the third ventricle, separated from it by a thin periventricular zone. As in rodents, there is a ventral retinorecipient zone in the monkey SCN that contains a large population of VIP+ neurons. Among the neurons, and surrounding them, is a dense plexus of VIP+ axons which extends out of the SCN into adjacent anterior hypothalamic area. The VIP+ population is surrounded by a smaller group of VP+ neurons that form a cap over the VIP+ neurons. There also is an associated VP+ axonal plexus but very few such fibers enter the zone of VIP+ neurons. The zone of RHT fiber termination also receives a dense plexus of NPY+ axons that are thinner and have smaller varicosities than the intrinsic hypothalamic NPY+ plexus that surrounds the SCN. Presumably the NPY+ plexus derives from NPY+ neurons in the IGL. There are a few neurotensin (NT+)-containing neurons with a sparse plexus in the monkey SCN. Thus, the organization of the monkey SCN is very similar to that in the rat.

5. <u>Human Lateral Geniculate Complex.</u> As in the monkey, the human lateral geniculate is dominated by the laminated DLG, with a large pregeniculate cell group medial to the DLG and smaller cell groups dorsal and lateral to the DLG. Also as in the monkey, there is a component of the dorsal and lateral cell groups that contains darkly staining neurons in Nissl material, is continuous with the thalamic reticular nucleus and which we conclude is the homologue of the cat perigeniculate nucleus. There are numerous NPY+ neurons in the pregeniculate nucleus dorsal and medial to the DLG. These are associated with a dense axonal plexus that extends throughout the entire nucleus. There are also extensive ENK+ and SP+ plexuses that extend over most of the nucleus, with the SP+ plexus less prominent dorsally and medially. Thus far we only have been able to examine 6 lateral geniculates as this is an area, unlike hypothalamus, routinely taken by neuropathologists for study. In addition, the ones we have examined have had variable fixation so that we need to stain more material to reach conclusions. We expect to be able to obtain better material in the future.

6. <u>Human RHT - Di I Studies.</u> Over the past two years we have studied 8 hypothalami in which Di I was placed in the optic nerve or the optic chiasm in close proximity to the SCN. With optic nerve placement, there is minimal transport along the nerve and none to the vicinity of the SCN, even with prolonged application, up to several months. With optic chiasm placement, we observe some fluorescent axons along the base of the SCN as reported by Stopa et al (1992) but no clear labeling in the nucleus even with prolonged exposure (up to 8 months at elevated temperature of 37°C.). Apparently Di I does not diffuse well along membranes of myelinated fibers in the adult nervous system. This has been reported to me by other investigators who have attempted to use Di I in the adult brain.

7. <u>Human SCN.</u> In the first part of this investigation we reanalyzed data obtained between 1978 and 1985. In that time a series of hypothalami was collected from the San Diego VAMC, University Hospital at Stony Brook and the Northport VAMC autopsy services. In addition, a series of brains at the Yakovlev Collection, Armed Forces Institute of Pathology, Washington, D.C., was examined. All of these had been embedded in celloidin or paraffin, cut in the coronal plane and stained with a Nissl stain. It had been our conclusion that the material was not informative. As in the older literature (cf Braak and Braak, 1987, for review), we had found an evident SCN in some brains and failed to find it in others. In the light of recent information, and particularly our analysis of the SCN using immunocytochemistry, it seemed worthwhile to analyze this material, including photomicrographs from the Yakovlev Collection brains, again. This was done from a simple perspective: Was the SCN discernable as a discrete nucleus in the material or not? That is, could one clearly identify an SCN with a distinctive cell population and clear boundaries in the material? The result is surprising and interesting. In brains obtained from late

gestation fetuses (33-36 weeks), newborns and young individuals to approximately age 50, the SCN is virtually always identifiable as a discrete nucleus with clear boundaries (Table I). From age 50-90, it is sometimes evident and sometimes not evident in the material. In the Nissl material, the difference between the brains in which the SCN can be easily identified and those in which it is not identified is in the general cellularity in the region.

TABLE I
THE HUMAN SUPRACHIASMATIC NUCLEUS (SCN)
IN NISSL STAINED MATERIAL

SOURCE OF BRAINS	N	AGES	SCN EVIDENT	SCN NOT EVIDENT
San Diego VAMC	27	55-90	12	15
Northport VAMC	12	58-86	5	7
University Hospital Stony Brook	5	33-38 weeks Gestation	5	0
Yakovlev Collection AFIP	4	Newborn	4	0
	7	2.5 months-11 years	7	0
	6	30-56	6	0
	5	63-88	1	4
	2	92 + 98	2	0

All brains were obtained at routine autopsy with a postmortem interval of 4 - 48 hours, fixed in neutral buffered formalin, embedded in paraffin or celloidin with coronal sections stained with thionin or cresyl violet. The specimens from the VA Medical Centers were all males. Those from other centers were approximately equally divided between male and female.

We have completed analysis of 22 hypothalami prepared for immunocytochemistry, including quantitative analysis. Sections are routinely stained for VIP, VP, NPY and NT. This analysis has revealed several interesting aspects of the human SCN. First, in contrast to what is found in Nissl material, the SCN is always evident as a distinct nucleus in immunocytochemical material. Second, it appears as the first component of the hypothalamus to be found in a rostrocaudal set of coronal sections. As soon as the rostral third ventricle is evident behind the lamina terminalis, the SCN appears as small clusters of cells immediately above the optic chiasm. The lateral portion of the lamina terminalis extends above the SCN. More caudally, as the SCN expands in size, the lamina terminalis is replaced by columns of preoptic-anterior hypothalamus extending dorsally, between the ventral portions of the cerebral hemispheres to meet in the midline at the organum vasculosum lamina terminalis. Third, the human SCN is characterized by four separate populations of neurons that have different peptide content. These neuronal populations have a different distribution in the nucleus. However, each population is represented by neurons at the rostral boundary of the nucleus. A quantitative analysis of the peptide-containing neurons is shown in Table 2. The smallest population is VIP+ neurons. These are present in the ventral portion of the nucleus and rostrally there are a number of VIP+ neurons present in the optic chiasm, embedded in pockets of neuropil. Within the body of the SCN, the VIP+ neurons are associated with a dense axonal plexus that extends throughout the rostral two-thirds of the SCN and the plexus continues beyond it into the retrochiasmatic area. We assume that, like in the monkey and rodents, the VIP+ neurons in the human represent a marker for the general area of termination of RHT projections. The next largest population is a unique human group, NPY+ neurons. In contrast to all other mammals, the human SCN contains a population of NPY+ neurons that overlaps the VIP+ group but extends dorsally beyond it in the center of the SCN. Among the NPY+ neurons are scattered coarse fibers and varicosities and a fairly dense plexus of very fine fibers and small varicosities. These are very similar in morphology to GHT projections in other mammals, particularly the cat and monkey. Since the human IGL does contain a NPY+ plexus, it would seem likely that at least some of these fine fibers originate in the IGL. There would appear to be three possibilities. First, both the IGL and the local SCN NPY+ neurons could contribute to this plexus. This would appear to be the most likely type of organization. Second, the NPY+ neurons in the IGL contribute the SCN plexus and the SCN NPY+ neurons project elsewhere. Third, the IGL NPY+ neurons project only within IGL and the SCN NPY+plexus is entirely from the intrinsic neurons. There is no way at present to distinguish between these possibilities. Surrounding the SCN NPY+ neurons is very dense plexus of NPY+ axons and terminals that is a continuation of the anterior hypothalamic NPY+ plexus into the "outer shell" of the SCN. A similar pattern is seen in other mammals.

TABLE 2
IMMUNOCYTOCHEMICAL ANALYSIS OF THE
HUMAN SUPRACHIASMATIC NUCLEUS

TOTAL	NEURON COUNTS				LENGTH	VOLUME
Neuron Number*	VIP	VP	NPY	NT	(mm)	(mm^3)
31,200 ± 1,088	3,638 ±383 (2226-5365)	7,447 ±559 (3915-10460)	5,029 ±339 (2870-7524)	15,086 ±878 (10494-20115)	3.9± 0.8	2.2± 0.6

* Total neuron number is calculated from the Sum of the VIP, VP, NPY and NT neuron counts. These data are presented as mean ±S.E.M. with the range of counts shown in parentheses. The quantitative analysis was made from 22 brains, 12 male and 10 female, ages 29-89.

There are two other populations of intrinsic SCN neurons, a VP+ one and a NT+ one. The VP+ neurons, like those in other mammals, form a cap around the VIP+ area. The plexus extends beyond the SCN borders but becomes obscured by VP+ fibers associated with the paraventricular nucleus. The NT+ neurons are the most numerous group, approximately 50% of the total, immunocytochemical-identified SCN neuron population (Table 2). NT+ neurons, and a dense axonal plexus, are present throughout the SCN except for a small area centrally in the caudal part of the nucleus. The SCN is surrounded by a very dense plexus of NT+ axons with occasional NT+ neurons in the anterior hypothalamus rendering it impossible to trace the projection of the SCN NT+ neurons. We have used antisera to galanin, CGRP, somatostatin, cholecystokinin, serotonin (5HT), SP and GAD- GABA in these studies as well as those noted above. As might be expected, we do not observe GABA immunoreactivity nor does the GAD antiserum provide a reproducible or informative pattern. There are no 5HT-immunoreactive structures in the human SCN but, as there are only very scattered 5HT+ fibers in other areas, this more likely represents a failure of preservation of the antigen than an absence of the innervation. No somatostatin- or cholecystokinin-immunoreactive structures are noted in the SCN. There are nearby somatostatin-containing neurons in the periventricular zone so that the absence of positive structures in the SCN is probably a significant negative finding. In all brains studied thus far, we find scattered galanin- and CGRP-immunoreactive axons in the central SCN. In adjacent hypothalamus there is dense galaNin staining but only scattered CGRP+ axons. The immunoreactive galanin and CGRP axons overlap the zone of the VIP+ neurons. Of particular interest, we find a large number of SP+ axons that exactly overlays the area of the VIP+ neurons. This suggests that, like the rat (Takatsuji et al, 1991), the human has SP-containing ganglion cells that project to the SCN through the RHT.

Fourth, there is substantial variation among individual brains. This is true in the number of neurons (Table 2) and in the shape of the nucleus. In some brains the nucleus is quite short in the dorsoventral dimension and in others it is quite long. This reflects, in large part, the location and exact shape of the optic chiasm. Whether differences in cell number have a functional consequence or not requires further analysis.

8. <u>SCN Efferents.</u> In prior work, we found that the pattern of SCN efferents in the rat, as shown by Watts et al (1987), is nearly exactly mimicked by VIP+ axons that leave the nucleus. The principal projection is into the anterior hypothalamic area extending from the SCN dorsally to the zone underneath the paraventricular nucleus (PVN). In VIP+ material, we see a similar pattern in the monkey; there is a dense plexus of fibers along the lateral border of the PVN. In contrast to the rat, however, there is a sizable extension of fibers into the PVN laterally and a more extensive plexus in the anterior hypothalamic area. At present we are limited in this analysis because our own monkey material has focused on the RHT projection using the CT-HRP method which does not allow good immunohistochemistry. The material obtained from other sources has been restricted to the SCN region but we now have promises of access to more extensive material. Similarly, with the human material, we have only recently had access to the material from the Suicide Center which is the only material with good VIP+ axon staining. This is also has been limited but we will have more access to this material in the future.

Publications.

1. Moore, R.Y. The organization of the circadian timing system. In Swaab, D. (Ed). The Human Hypothalamus in Health and Disease, Elsevier, Amsterdam, 1992, pp. 101-117.

2. Moore, R.Y. Organization of the human circadian system. J. Biol. Rhythms, in press.

3. Moore, R.Y. Retinohypothalamic projections and the organization of the suprachiasmatic nucleus in the macaque monkey. In preparation. To be submitted to the Journal of Neuroscience.

4. Moore, R.Y. and Speh, J.C. *Intergeniculate leaflet and ventral lateral geniculate in the* macaque monkey. In preparation. To be submitted to Visual Neuroscience.

5. Weis, R. and Moore, R.Y. Organization of the human paraventricular nucleus. In preparation. To be submitted to Cell and Tissue Reseach.

6. Moore, R.Y. and Speh, J.C. The human retinohypothalamic tract is demonstrated by substance P immunoreactivity. In preparation. To be submitted to Brain Research.

7. Speh, J.C. and Moore, R.Y. Retinohypothalamic projections in the macaque monkey. Society for Neuroscience Abstracts. 17: 670, 1991.

Inventions, Patents. None.